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Divergent contributions of regulatory T cells to the pathogenesis of chronic hepatitis C

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Abbreviations: DCs, dendritic cells; FoxP3, transcription factor forkhead box P3; HCV, hepatitis C virus; IFN, interferon; IL, interleukin; iT_{reg} cell, inducible regulatory T cell; nT_{reg} cell, natural regulatory T cell; T_{eff} cells, effector T cells; TGF-β, transforming growth factor-beta

Hepatitis C virus, a small single-stranded RNA virus, is a major cause of chronic liver disease. Resolution of primary hepatitis C virus infections depends upon the vigorous responses of $CD4^+$ and $CD8^+$ T cells to multiple viral epitopes. Although such broad $CD4^+$ and $CD8^+$ T-cell responses are readily detected early during the course of infection regardless of clinical outcome, they are not maintained in individuals who develop chronic disease. Purportedly, a variety of factors contribute to the diminished T-cell responses observed in chronic, virus-infected patients including the induction of and biological suppression by $CD4^+FoxP3^+$ regulatory T cells. Indeed, a wealth of evidence suggests that regulatory T cells play diverse roles in the pathogenesis of chronic hepatitis C, impairing the effector T-cell response and viral clearance early during the course of infection and suppressing liver injury as the disease progresses. The factors that affect the generation and biological response of regulatory T cells in chronic, hepatitis C virus-infected patients are discussed.

Introduction

Hepatitis C virus (HCV, a small single-stranded RNA virus) is the cause of hepatitis C, the most common blood-borne infection in the world; an estimated 180 million people globally, ~3 million in the US, are chronically infected.[1,2] The RNA genome consists of a large open reading frame that encodes an approximate 3,000 amino acid polyprotein precursor that is cleaved by viral and cellular proteases to yield three structural proteins [core, envelope 1 (E1) and E2] and seven non-structural proteins, which are involved in viral synthesis.[3] HCV is primarily transmitted via contact with infected blood or bodily fluids. The risk of contracting hepatitis C from contaminated blood or blood products in the US is negligible due to mandatory and reliable blood screening.[4] Despite a safe blood supply, however, isolated nosocomial/iatrogenic infections continue to occur, emphasizing the need for enforcement of universal precautions and proper sterilization of medical devices.[4] Individuals at highest risk of infection in the US today are injection drug users.[5] In contrast, a large percentage of the population in many developing countries is infected owing to substandard infrastructure and technology required to provide a safe blood supply.[6]

The majority of HCV-infected patients develops chronic disease; only an approximate 20% resolve infection spontaneously.[2] A variety of factors such as age, sex, ethnicity, human immunodeficiency virus or hepatitis B co-infection, and host genetics (e.g., IL28B gene variants and HLA haplotype) affect spontaneous clearance of HCV.[7-10] Standard treatment of unresolved infections, consisting of the combined administration of PEGylated interferon (IFN) and ribavirin over an extended period of 24 to 48 weeks, is often accompanied by significant side effects. Moreover, only 45–50% of treated patients infected with HCV genotype 1 (the primary etiologic agent of hepatitis C in the US) demonstrate a sustained virologic response following treatment withdrawal.[11] Additional treatment approaches that combine protease inhibitors, e.g., boceprevir and telaprevir, with ribavirin and PEGylated IFN result in sustained virologic responses in up to 80% of patients infected with HCV genotype 1, though the severity of accompanying side effects can also increase dramatically.[12-14]

The vigorous responses of HLA class I- ($CD8^+$) and class II-restricted ($CD4^+$) T cells to numerous viral epitopes derived from both structural and nonstructural proteins are required for the resolution of primary HCV infections.[15] Such broad responses are readily detected during the early course of infection despite clinical outcome, but subside in patients who later develop chronic disease.[16] While patients who spontaneously clear infection continue to exhibit sustained, proliferative responses to a broad array of class I- and class II-restricted epitopes, chronically infected individuals respond to only a limited number.[17] Purportedly, multiple factors contribute to the reduction in T-cell responses found in patients with chronic infections, notably, the induction of and biological suppression by $CD4^+FoxP3^+$ regulatory $T_{(reg)}$ cells.[18-22]

Regulatory T Cells

Background. $CD4^+$ regulatory $T_{(reg)}$ cells constitute one of the major mechanisms underlying immunological homeostasis and

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self-tolerance.[23] T_{reg} cells are characterized by the constitutive expression of the transcription factor forkhead box P3 (FoxP3) and interleukin (IL)-2 receptor α chain (CD25) on the cell surface. FoxP3 mutation and T_{reg} cell dysfunction result in fatal immunopathology and autoimmune disease in both mice and men.[24-26] T_{reg} cells also moderate effector immune responses to infectious diseases that, if sustained at elevated levels, can lead to serious host tissue and organ damage.[27] Although a key factor in the maintenance of immune homeostasis, T_{reg} cells can also suppress effective immune responses to autologous tumors (e.g., hematological malignancies and metastatic melanoma) and promote persistent infections by a wide variety of microbial pathogens.[27-29]

T_{reg} cell phenotype. Two distinct T_{reg} cell subsets, classically distinguished by site of origin, are described in the literature. Natural (n)T_{reg} cells are generated by high-avidity selection in the thymus; inducible (i)T_{reg} cells, on the other hand, derive from conventional ($CD4^+CD25^-FoxP3^-$) T cells in the periphery following stimulation.[30-32] nT_{reg} cells can induce "infectious tolerance" by converting conventional T cells into iT_{reg} cells via two primary methods: cytokine (IL-10, IL-35 or TGF-β)-dependent and dendritic cell (DC)-mediated, cytokine-independent mechanisms.[33,34] Purportedly, nT_{reg} and iT_{reg} cells possess complementary immune functions: prevention of autoimmunity and maintenance of a non-inflammatory environment, respectively.[31]

Notably, no specific marker defines T_{reg} cells or differentiates nT_{reg} and iT_{reg} cell subsets. While FoxP3 expression is a common attribute of both subsets, conventional human T cells lacking immunosuppressive capacity can also express FoxP3 transiently following activation.[32] Moreover, despite the near exclusive expression of CD25 by nT_{reg} cells in naïve mice, CD25 is expressed by a much more heterogeneous T-cell population in humans.[32] Recent studies report the high level expression of neuropilin-1 on the surface of nT_{reg}, but not iT_{reg}, cells in mice, enabling differentiation and separation of these two subsets.[35,36] Activated human $FoxP3^+$ T_{reg} cells that express high suppressive activity are also distinguished by presence of glycoprotein A repetitions predominant (GARP, or LRRC32), a cell surface transmembrane protein that contains leucine-rich repeats.[37-40] GARP mRNA is specifically expressed by $CD4^+CD25^{hi}$ T_{reg} cells, and is rapidly upregulated following T-cell receptor engagement.[37,38] GARP anchors transforming growth factor (TGF)-β to the cell surface conferring increased suppressive activity and the ability to induce infectious tolerance.[39] Lastly, cell surface expression of ectonucleotidase, CD39, distinguishes activated, effector memory T_{reg} cells capable of abrogating DC maturation and T cell-dependent cytotoxicity.[41]

T_{reg} Cell Function

Contact-independent mechanisms. Activated T_{reg} cells are able to suppress the activity of a variety of immune cell types, i.e., both $CD8^+$ and $CD4^+$ T cells, NK cells, NKT cells, B cells, macrophages and DCs.[42-46] Multiple mechanisms contribute to this suppressive activity although it is widely believed that nT_{reg} cell-mediated suppression is dependent upon direct, cell–cell contact.[46] The synthesis of inhibitory cytokines constitutes a principal contact-independent mechanism by which T_{reg} cells in general suppress T_{eff} cell activity (**Fig. 1**). Both the soluble and membrane-bound forms of TGF-β, for example, play key roles in inducing and/or maintaining iT_{reg} and nT_{reg} cells, and in suppressing conventional effector $T_{(eff)}$ cell activation.[45,47,48] Similarly, IL-10 plays a critical role in suppressing $CD4^+$ T_{eff} cell responses to a variety of pathogens used in animal models, as well as those that contribute to human disease.[27]

The constitutive, high-level expression of CD25 (IL-2 receptor α chain) constitutes an additional contact-independent mechanism underlying T_{reg} cell-mediated suppression. T_{reg} cells produce relatively low levels of IL-2 and, as such, require an exogenous source of IL-2 in order to proliferate and survive.[49] As a consequence of the rapid consumption of IL-2 by T_{reg} cells, T_{eff} cell populations are deprived of the cytokine necessary for activation.[49] The cell surface expression of CD39 and CD73 ectonucleotidases constitutes another mechanism by which T_{reg} cells disrupt the metabolic activity of T_{eff} cells.[50] The activity expressed by these molecules abrogates the proinflammatory response of T_{eff} cells by rapidly degrading extracellular ATP released by neighboring, activated or damaged cells.[50] Additionally, adenosine generated as a byproduct of ATP degradation further suppresses T_{eff} cell function by binding A2A receptors expressed on the cell surface and inducing T cell anergy.[50-52]

Contact-dependent mechanisms. A number of contact-dependent mechanisms also facilitate the ability of T_{reg} cells to suppress T_{eff} cell function. For example, T_{reg} cells can exhibit cytotoxic activity and induce T_{eff} cell apoptosis dependent upon the production of granzyme A, granzyme B and perforin.[48,53] In addition, cell-surface galectin-1 appears to contribute to the immunosuppressive activity of T_{reg} cells.[54] A member of a highly conserved family of β-galactosidase-binding proteins, galectin-1 inhibits proliferation and promotes apoptosis of activated T_{eff} cells.[54]

Apart from regulating T_{eff} cell function directly, T_{reg} cells can inhibit the maturation and immunostimulatory activity of DC and, consequently, suppress the activity of conventional T cells indirectly.[48] T_{reg} cells can render DCs tolerogenic and limit their capacity to sensitize naïve T cells by a process termed trans-endocytosis whereby cytotoxic T lymphocyte-4 (CTLA-4) molecules expressed by T_{reg} cells capture, internalize, and degrade co-stimulatory molecules (i.e., CD80 and CD86) expressed on the surface of DCs.[55] Lymphocyte activation gene-3 (LAG-3 of CD223), a CD4 homolog capable of binding MHC class II molecules, further suppresses DC maturation and the expression of co-stimulatory molecules.[56,57] Lastly, neuropilin-1 expressed by nT_{reg} cells inhibits the sensitization of naïve $CD4^+$ T cells by promoting extended interaction between T_{reg} cells and immature DCs.[58] Undoubtedly, the suppressive functions of T_{reg} cells depend upon a combination of the mechanisms described.

T_{reg} Cells in Hepatitis C

The increased numbers of T_{reg} cells present in the liver and peripheral blood of chronic HCV-infected patients relative to patients

who spontaneously clear infection suggests that T_{reg} cells play a significant role in the pathogenesis of chronic hepatitis C.[18,19,27,59-63] In this regard, $CD4^+CD25^+$ T cells obtained from the peripheral blood of patients infected with HCV suppressed virus-specific proliferation and IFN-γ production by $CD4^+$ and $CD8^+$ T cell; depletion of $CD25^+$ cells including T_{regs} from the total peripheral blood mononuclear cell (PBMC) population enhanced the proliferation of cells that remained.[18,63] Whether the increased number of T_{reg} cells found in the livers of chronically-infected patients represents an virus-specific T_{reg} cell response or arises as a non-specific consequence of chronic inflammation and disease is not entirely clear.

HCV encodes T_{reg} cell epitopes. Researchers have identified T_{reg} cell epitopes present in both the structural and non-structural proteins synthesized by HCV.[20,59,60,64-66] Using HCV peptide-loaded MHC class II tetramers to label and quantify the cells, Ebimuna and coworkers reported HCV epitope-specific recognition by $FoxP3^+$ T_{reg} cells present in the peripheral blood of chronically infected patients.[64] Moreover, a number of studies now report an increase in $FoxP3^+CD25^{high}$ cells that resemble nT_{reg} cells and express an anergic cytokine profile following stimulation with HCV derived epitopes.[59,61,64] Comparing FACS-sorted $CD4^+CD25^{high}$ T cells (nT_{reg} phenotype) derived from uninfected and HCV-infected individuals, however, one study found only minimal differences in global gene expression.[64] In contrast, other investigators reported an increase in HCV epitope-specific T_{reg} cells that resembled iT_{reg} cells phenotypically and exhibited both IL-10- and TGF-β-dependent suppressive activity.[60,65] Regardless, a general consensus supports the heterogeneous nature of the expanded T_{reg} cell population found in chronic, HCV-infected patients (i.e., composed of both nT_{reg} and iT_{reg} cell subsets). The factors that contribute to this expansion are neither well understood nor characterized. Conceivably, the initial response of nT_{reg} cells to infection promotes the conversion of conventional T cells to iT_{reg} cells and subsequent suppression (i.e., infectious tolerance).[34]

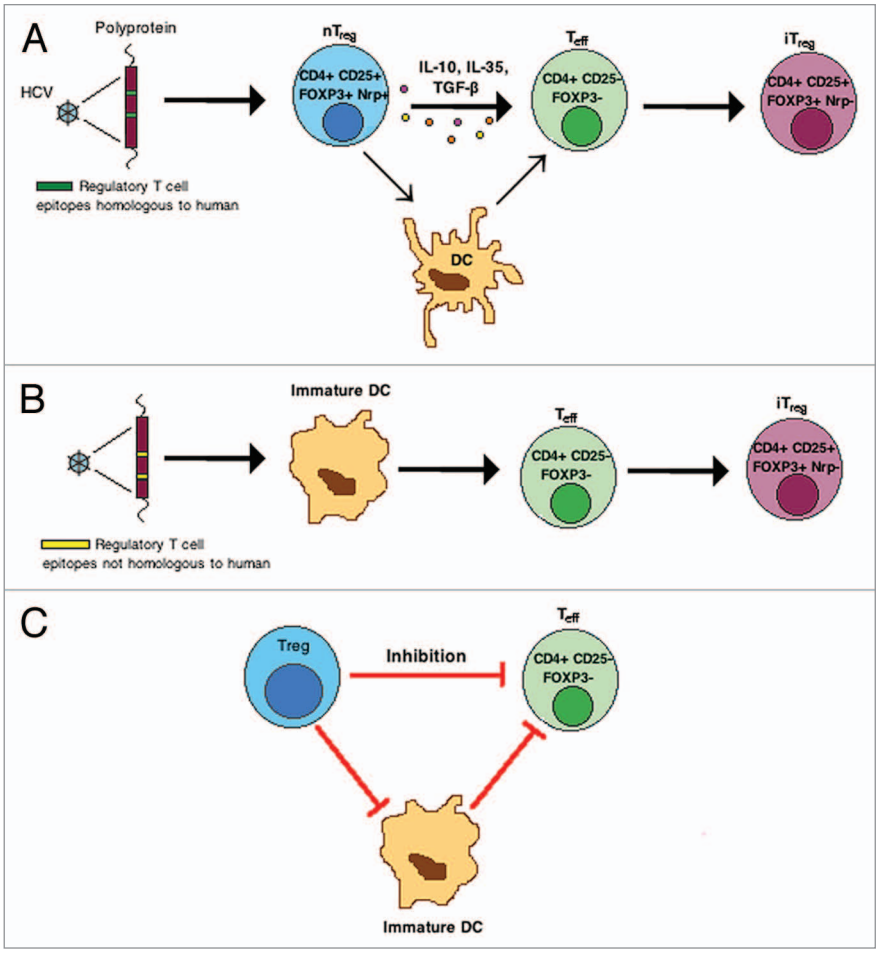

Figure 1. Increases in both the number and function of T_{reg} cells have been implicated in the pathogenesis of chronic hepatitis C. Virus-associated regulatory T cell epitopes, homologous to peptide sequences found in the human plasma proteome, induce nT_{reg} cell activation, conversion of T_{eff} to iT_{reg} cells and infectious tolerance (**A**). Viral epitopes lacking human homology, which are presented by immature DCs, elicit additional HCV-specific iT_{reg} cells (**B**). T_{reg} cells inhibit T_{eff} cell function by direct, contact-dependent and -independent mechanisms and by indirect mechanisms that affect DC maturation and/or immunostimulatory activity (**C**).

HCV T_{reg} cell epitopes and human homology. The expressed function of nT_{reg} cells is to suppress autoimmunity and the response to self antigens.[23-26] Activation of T_{reg} cells subsequent to HCV infection suggests the existence of viral epitopes that exhibit homology to self and the ability to simulate these nT_{reg} cells and viral persistence.[59,67-70] Indeed, Kanduc and colleagues reported that 3003 unique pentapeptides comprised the HCV polyprotein; of these, 2760 pentapeptides occurred a total of 46,731 times (including matches/repeats) in 20,269 proteins that comprised the human proteome.[68,70] Furthermore, a BLAST search and JanusMatrix analysis (a new computer algorithm, which identifies epitopes that are highly cross-reactive on the T cell receptor aspect) revealed extensive homology between a number of published T_{reg} cell epitopes derived from HCV structural and non-structural proteins and sequences encoded by the human genome (Moise et al., manuscript submitted) (Losikoff et al., manuscript in preparation).[71] These epitopes induced the response of T_{reg} cells present in the peripheral blood of chronic HCV-infected patients, but not in blood obtained from HCV non-infected individuals.[22,59,71] Taken together, these findings suggest that viral epitopes, homologous to peptide sequences constituting the human proteome, influence the pathogenesis of chronic hepatitis C by inducing the expansion of the iT_{reg} cell population and infectious tolerance.[62,72]

An evolving body of research demonstrates the import of the human microbiome in shaping the immune response to viral

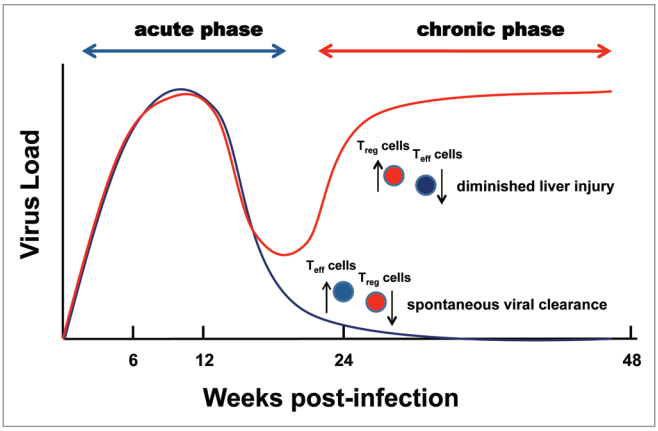

Figure 2. Contrasting contributions of T_{reg} cells to the pathogenesis of chronic hepatitis C. An increased ratio of T_{reg} to T_{eff} cells impairs spontaneous viral clearance and suppresses liver injury and the pathogenesis of chronic hepatitis C as the disease progresses.

pathogens.[73] Recent studies found the microbiome can induce the differentiation and expansion of iT_{reg} cells that exhibit a unique T cell receptor repertoire specific for commensal bacteria.[74,75] Conceivably, homology between viral epitopes and T_{reg} cell epitopes associated with the human microbiome represents an additional mechanism underlying the expansion of T_{reg} cells in chronic HCV.

T_{reg} Cells Impair Viral Clearance

Several cross-sectional studies demonstrated a strong correlation between chronic HCV infection and increased T_{reg} cell frequencies.[18,63,64] The specific contribution of T_{reg} cells to viral persistence, however, could not be elucidated due to the occurrence of infection at some previous, undetermined date. Furthermore, two longitudinal studies failed to demonstrate a significant difference in the frequency or function of T_{reg} cells in the blood of patients acutely infected with HCV regardless of clinical outcome (i.e., whether the virus was eventually cleared or chronic disease developed).[61,76] In one of these studies, T_{reg} cells obtained from patients who subsequently developed chronic disease demonstrated significantly more suppressive activity than did T_{reg} cells obtained from patients who spontaneously cleared infection.[61] As a consequence, the authors concluded that maintenance of an elevated T_{reg} cell population promoted the development of chronic hepatitis C.

Recently, Cusick and coworkers described the emergence of a variant of an immunodominant HCV epitope during the course of chronic HCV infection.[66] This variant elicited a T_{reg} cell population that suppressed a T_{eff} cell response to the cognate, but not an unrelated, peptide. This finding suggests that variant, MHC class II-restricted viral epitopes arise as a consequence of immune pressure during the course of chronic HCV infection. Rather than induce the activity of conventional $CD4^+$ T cells, these variant epitopes elicit an epitope-specific T_{reg} cell response that diminishes the $CD4^+$ T cell help required for viral clearance. Thus, in cases of chronic disease, T_{reg} cells impair T_{eff} cell function and promote viral persistence by inhibiting virus-specific helper T cell activity.

T_{reg} Cells Suppress Chronic Hepatitis C-Associated Liver Injury

In addition to inhibiting effective immune responses and promoting persistent infections by a wide variety of pathogenic microorganisms, T_{reg} cells moderate T_{eff} cell responses that could otherwise lead to serious host tissue and organ damage.[18,21,27,28,77-79] This is evidenced in patients with chronic hepatitis C by an inverse correlation between the level of T_{reg} cell activity found in the peripheral blood and the extent of liver injury.[18,78,80] Both the serum alanine aminotransferase concentrations and the histopathologic scores of liver biopsies were higher in chronic HCV-infected patients who exhibited a relative reduction in T_{reg} cell activity suggesting that the expanded T_{reg} cell population ameliorated excessive pathology and tissue damage. The balance between T_{reg} cells and $CD8^+$ T_{eff} cells appears to be a crucial factor. Sturm and colleagues reported a strong inverse correlation between the severity of liver fibrosis and the accumulation of $CD4^+FoxP3^+$ T_{reg} cells in close contact with $CD8^+$ T cells in necro-inflammatory sites in patients with chronic hepatitis C.[21] The ratio of $FoxP3^+$ to $CD8^+$ T cells was elevated in the early stages of fibrosis, and reduced substantially in cirrhotic livers. The authors also reported a link between IL-10 and TGF-β message expression in the liver and presence of $FoxP3^+$ and $CD8^+$ T cells suggesting that these anti-inflammatory cytokines served both to maintain the T_{reg} cell pool and inhibit the production of pro-inflammatory cytokine (e.g., TNF-α and IFN-γ) by CD8 T_{eff} cells.[21]

Thus, an equilibrium between T_{reg} cells and T_{eff} cells that permits both viral clearance and protection from HCV-related hepatopathology is critical for an optimal response to HCV infection.[21,77] In cases of chronic disease, however, the elevated presence of T_{reg} cells appears to benefit both the pathogen (i.e., viral persistence) and the host (i.e., prevention of immunopathogenesis).[21,79] In this regard, it is interesting to note that increased numbers of $CD25^+FoxP3^+$ T_{reg} cells were found in the livers of patients who underwent successful chemotherapy, suggesting that the continued presence of T_{reg} cells moderated immunopathology despite viral clearance. These divergent roles of T_{reg} cells during the development of chronic hepatitis C are illustrated in **Figure 2**.

Impaired DC Function Promotes the Generation of T_{reg} Cells

Whether HCV infection elicits the response of T_{reg} cells directly or indirectly (dependent upon the intermediary role of another cell type) remains to be determined. Accumulating evidence suggests, however, that DCs play a key role in the expansion of T_{reg} cells during the pathogenesis of chronic disease. DCs are professional antigen presenting cells characterized by the potent capacity to elicit primary T cell responses.[81] While there is no general consensus regarding the effects of HCV infection on DC function, it is often agreed that chronically infected patients

have significantly fewer DCs circulating in the peripheral blood, which correlates inversely with the severity of liver disease.[82-86] Conversely, increased numbers of DCs are found in the liver of patients chronically infected with HCV.[85,87-89] Regardless of their physiologic distribution (i.e., peripheral blood vs. liver), it is frequently suggested that the DCs are functionally impaired in patients with chronic hepatitis C.[83,89-98] These impairments include lowered expression of HLA-DR and costimulatory molecules (e.g., CD86), decreased allostimulatory activity and the ability to secrete IFN-α and IL-12 and increased IL-10 production and the ability to prime T_{reg} cells.[83-85,89,90,92-96,99-103] Additionally, it was reported that DCs derived from chronically infected patients remain phenotypically immature and lack the capacity to upregulate the expression of maturation or costimulatory marker following stimulation.[91,97]

Mechanisms that underlie impaired DC function. The specific mechanisms underlying DC dysfunction in patients chronically infected with HCV remain to be clarified. Recent studies suggest, on the one hand, that DCs are susceptible to HCV infection.[104,105] The interaction between envelope glycoproteins E1 and E2 and DC-SIGN, a C-type lectin expressed by DCs, mediates the ingestion of HCV viral particles.[105] This finding supports the speculation that, even in the absence of a productive infection, E1 and E2 bound to DC-SIGN transmit a signal that promotes T_{reg} cell formation and tolerance. Other structural and non-structural viral proteins further impair DC function by decreasing costimulatory molecule and HLA expression, reducing cytokine production, preventing TLR signaling and diminishing allostimulatory activity.[106] As described above, T_{reg} cells can also inhibit DC maturation and immunostimulatory capacity by a variety of mechanisms and, thereby, suppress the response of conventional T cells.[48] A growing body of evidence supports the critical role of DCs in the induction and maintenance of T_{reg} cells during chronic HCV infection, regardless of the specific mechanisms that contribute to a diminution in DC function.

DCs and immunological tolerance. DCs play a key role in establishing and maintaining immunological tolerance to both foreign and self antigens.[107,108] Interactions between T_{reg} cells and DCs in the prototypic, tolerogenic environment maintained in the liver foster the development of chronic infectious diseases such as viral hepatitis.[109] While it is generally agreed that immature DCs promote T_{reg} cell function, the underlying mechanisms remain to be fully delineated.[107-111] Notably, DCs obtained from human liver principally exhibit an immature phenotype characterized by low or negligible expression of cell-surface costimulatory molecule (e.g., CD40, CD80, CD83 and CD86).[109] Relative to DCs purified from the peripheral blood, DCs derived from liver also produce lower levels of proinflammatory cytokine (i.e., TNF-α, IL-1β and IL-6) and significantly higher levels of IL-10 following stimulation. Moreover, the IL-10-dependent production of $CD4^+CD25^+FoxP3^+$ T_{reg} cells was greater in co-cultures that contained naïve, allogeneic $CD4^+$ T cells and liver DCs, compared with co-cultures that contained DCs derived from blood.[110] Similar results were obtained when allogeneic T cells and DCs obtained from chronic, HCV-infected patients were co-cultured, i.e., greater expansion of the T_{reg} cell population than observed in co-cultures that contained DCs obtained from healthy individuals.[103]

Summary and Clinical Implications

Eighty to 85% of the patients in the US infected with HCV develop chronic disease.[2] Persistent viral infection correlates with increased T_{reg} cell number and function in the tissues of HCV-infected patients. While triple drug therapy (e.g., boceprevir or telapervir administered in conjunction with interferon and ribavirin) increases the sustained virologic response, the adverse side effects are often severe, the costs are extraordinarily high, and a significant portion of treated patients remains infected.[12-14] Additional approaches, e.g., therapeutic vaccination, are urgently needed. To date, four vaccine strategies have demonstrated varied and only limited success in clinical trials: recombinant protein, peptide, genetic or DNA-based and vector-mediated.[112,113] Recent evidence suggests that viral epitopes may activate nT_{reg} cells and, subsequently, induce expansion of the iT_{reg} cell population and immunosuppression via the direct effects of T_{reg} cells on T_{eff} cell activity or by indirect effects dependent upon intervening DCs. DCs that exhibit an immature phenotype, in turn, can promote the expansion and activity of T_{reg} cells and, thus, play a key role in exacerbating the pathogenesis of chronic hepatitis C. As a consequence, future efforts to develop a therapeutic vaccine must employ strategies to avoid incorporating epitopes that promote T_{reg} cell activity.

In the absence of a therapeutic vaccine, a full understanding of the response of T_{reg} cells to HCV infection could lead to the development of treatments capable of sustaining T_{eff} cell-mediated immunity while limiting tissue damage.[114] In this regard, continued research efforts are needed to determine the most effective means of manipulating the T_{reg} cell response in a clinical setting.[18] Progress in understanding the role of T_{reg} cells and the factors that influence protective immunity to hepatitis C are hampered, however, by the lack of a good rodent model and the ability of HCV to infect humans and chimpanzees only.[115]

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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